The use of Elisa as a complementary tool for bovine tuberculosis control in Brazil

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Abstract

The detection of infected animals is one of the main factors involved in tuberculosis control and, with some variations, is performed through intradermal tuberculin tests. Nevertheless, skin test-negative animals may be infected and represent an important threat to bovine tuberculosis eradication programs. Despite this well-known phenomenon, ELISA testing did not become a routine in tuberculosis control programs. Our purpose was to describe field applications of ELISA in the detection of anergic animals in Rio de Janeiro, Brazil. From 18 herds involved on a tuberculosis control program, two presented skin test-negative infected animals what have delayed and compromised the success of the program, with severe economic losses. Infection in those animals was identified through ELISA and confirmed by isolation of *M. bovis* from lung lesions. Therefore they were considered to be the most probable source of continuing infection and responsible for the maintenance of the disease in their herds. Without the use of serological tests as ELISA those cows would probably stay in their herds perpetuating the disease and the eradication of the disease in those herds would become impossible. In conclusion, we suggest the use of ELISA as a valuable complementary tool in order to identify possible anergic cows that may be acting as reservoirs of the agent in the herd.

Introduction

Bovine tuberculosis is a worldwide disease. In Brazil, it is still frequent, mainly in dairy cattle[1]. In order to control this important threat to animal and public health and to reverse this situation, Brazilian Department of Agriculture has recently initiated a National Tuberculosis Control Program. This is a test-and-slaughter based program, using single cervical tuberculin test (SCTT) as the standard test and comparative cervical tuberculin test (CCTT) as the confirmatory method. Nevertheless, the intradermal tuberculin test presents some important limitations, mainly related to its sensitivity[2]. The use of the gold-standard diagnostic method, bacteriological culture, although desirable and indicated for specific situations, has also important limitations, mainly due to the limited number of specialized laboratories.

In some countries, the lack of overall success of field programs for disease eradication has resulted in the instigation of research to develop better diagnostic tests and vaccines, and to determine potential sources of infection, including wildlife [3].

The occurrence of infected animals that cannot be detected by skin testing has been reported[4,5]. Those animals stay in the herd maintaining the disease and compromising the success of the program. Those animals usually present advanced disease and since they are not identified by intradermal tests and quickly eliminated, they tend to shed viable *M. bovis* via the respiratory tract and to infect other cattle, making

eradication of the disease from the herd more difficult.

This is an important aspect to be considered when an extensive control program is under development, since it can delay and even compromise the success of the program. The possibility of using serological tests such as ELISA for detecting those anergic animals has already been suggested [6,5], but it is not officially recommended and therefore does not represent a routine for tuberculosis diagnosis. After previous work [6] where the use of ELISA for the detection of anergic animals has been suggested, some practical applications of its use have been raised. The purpose of that study was to demonstrate in practical situations the use of ELISA as a valuable complementary tool in field conditions for the control of bovine tuberculosis in Brazil.

Materials and Methods

Herds – Eighteen herds involved on a tuberculosis control program located in Rio de Janeiro, Brazil have been evaluated. Animals were tested by cervical comparative tuberculin test (CCTT) each 90 days. On two herds reactive animals kept arising and, since a possible lack of sensitivity of CCTT was considered, ELISA has been applied on those two herds. Herd A consisted of a 360 cow dairy herd with an undergoing tuberculosis control program based on CCTT. Any reactive cow was immediately (maximum 15 days) slaughtered and new cattle were not added to the herd for the last three years. Also, animals were not allowed to leave the farm and return from expositions or milk competitions. Herd B was a small dairy herd with 31 high-production cows bred in intensive management system. Cows were kept in a stable and allowed to walk for only four hours a day. The herd had been skin tested by SCTT for several times five years ago and reactive animals had been all culled. No animals had been acquired since that time.

After three negative skin tests, the herd was considered as tuberculosis-free and regular skin testing of the animals had been discontinued three years ago.

Tests - Comparative cervical tuberculin test using both bovine and avian PPD (CCTT) was performed according to official Brazilian standards, as previously described [1]. When applicable, necropsy procedures and bacteriological examination of lungs and lymph nodes was performed [1] using Löwenstein-Jensen culture medium with added pyruvate and Middlebrook 7H11 medium. Tubes were examined each week and 60-80 days after inoculation colonies of mycobacteria were obtained and identified bio chemically as *Mycobacterium bovis*. An ELISA for detection of antibody with reported sensitivity and specificity rates of 86·7% and 90·6% was performed following a previously described protocol [6]. Briefly, this protocol uses flat-bottomed polystyrene microtiter plates (Dynatech Labs., USA) Immulon 2 coated (1mg/well) with bovine tuberculin purified protein derivative (PPD; Tecpar, Brazil), blocked with 100 ml/well of 0.5% casein. A pool of eight positive sera (from culture-positive animals) and a pool of eight sera from cows originating from farms without tuberculosis and negative to intradermal reactions were used as controls. Serum samples and control sera were tested in duplicate and incubated with a 1:500 dilution of monoclonal anti-bovine IgG conjugated with alkaline phosphatase (Sigma) and absorbencies are read at 405 nm. Animals were considered as reactive when Optical Density (O.D.) was > 0.350. The mean absorbency values were calculated from the replicate wells for each serum, and samples showing coefficient values of greater than 15% were repeated. Final OD's represent the difference in absorbance between wells containing antigen minus wells lacking antigens.

Results

In the beginning of the control program

Herd A presented 42 (11.6%) reactive animals. All of them were identified and slaughtered. After 90 days, another CCTT was performed and 14 (4.4%) new cases were detected and culled. The number of reactive animals decreased progressively at each tuberculin testing performed on 90-days intervals till day 270, when four reactive animals were culled due to positive CCTT reactions. The expectation was to keep decreasing that number till the complete eradication of the infection, by elimination of infected animals harboring the agent.

Nevertheless, despite of the expectations and of elimination of all the CCTT-positive animals, 15 months after the beginning of the program four infected animals were detected by intradermal testing. No other infectious sources such as environmental or wildlife conditions were identified. Therefore, the hypothesis of the existence of at least one anergic animal among the skin-test negative animals was considered and ELISA has been applied. In order to avoid PPD stimulation of an antibody reaction, animals were bled for ELISA and skin-tested at the same day, 90 days after the last injection of bovine PPD.

Six animals presented an Optical Density (OD) > 0.350, the cut-off considered to that study, as previously recommended[6]. Four of them also presented CCTT-positive reactions at this time and were slaughtered. The remaining two cows (one with OD = 0.386 and one with OD = 1.504) were negative to intradermal tests but have been slaughtered and necropsied. The first animal presented no visible lesions and bacteriological processing of the lymph nodes and lung tissue was negative. This cow was considered as not infected and the result of ELISA interpreted as a false-positive reaction. The fact of the OD reading was close to the cut-off point was also considered to reach that conclusion. Nevertheless, the latest animal, a seven-year-old Holstein cow, presented several lung granulomatous lesions with caseous necrosis. Various tubercles and lymph nodes granulomatous lesions were also observed, including alterations observed in the uterus of the animal.

Caseous material and lymph nodes were stained and fast-acid bacilli were observed. Isolates of *Mycobacterium bovis* were recovered from lung tissues. This cow was infected and has been classified as anergic, constituting the most probable source of the infection on the herd. After the slaughtering of that animal, two more skin-reactive animals were detected in the following test, performed 90 days after the ELISA test, and were also culled. For over one year five CCTT tests have identified no new reactive animals. ELISA was performed on the entire herd 60 days after the last CCTT and all the animals presented low OD. Therefore, the herd was finally considered as tuberculosis-free.

Herd B was first considered to be a tuberculosis-free herd. When one cow presented leanness and coughing for two months, pleuropneumonia was suggested as the most probable diagnosis and antimicrobial therapeutics were unsuccessfully tried. The animal has been slaughtered and typical lesions of tuberculosis have been observed in the lungs. Tissue samples have been collected and *Mycobacterium bovis* was recovered from the lungs. After that finding, all the animals of the herd were skin tested and 16 (53.5%) cows presented reactive. The extremely high rate of infected animals was a great surprise, and can be explained by the intensive management care of the animals and by the absence of a regular test-and-slaughter program on the herd for the last three years.

The source of the infection was questioned, since no animal had entered the herd and all the present animals had presented previous three negative results at CCTT. No evidence of wildlife that could be responsible for the infection was identified. The possibility of the existence of at least one anergic animal among those non-reactive cows has raised. ELISA was performed on the entire herd and one cow

presented an OD of 0.696. This cow was 8 years old and was in the herd since 2 years of age, when new animals had been acquired by the last time. This cow had always been non-reactive to CCTT and was maintained in the herd after it was declared TB-free. When slaughtered, this animal presented extensive granulomatous lesions on the lungs and on mediastinal lymph nodes. Tissue samples were bacteriology performed and *Mycobacterium bovis* was recovered

After the culling of all CCTT reactive animals and also of that anergic animal, two animals presented CCTT positive reactions after 90 days and then no new cases were observed. The herd has been repopulated with CCTT and ELISA- negative animals and has been regularly tested for one year, with no positive animals.

Discussion

Several authors have suggested the use of combined cellular and humoral tests for the diagnosis of bovine tuberculosis but it is not a common procedure[8,9,5]. While the delayed-type hypersensitivity (DTH) reaction is indicative of infection or exposure, antibody formation appears to be more closely related to the extent of bacterial multiplication and antigenic load in the infected individual 2.

In a very comprehensive review about bovine tuberculosis, Pollock and Neill[3] discussed the occurrence of tuberculosis-associated anergy on cattle. Although antibodies are usually associated to disease progression, under certain circumstances in advanced, possibly disseminated tuberculosis, cattle may become anergic. Therefore, some animals have been found with typical disease at slaughter apparently without ever having responded to tuberculin skin testing[4].

The mechanisms of tuberculosis-associated anergy are not well understood. Interestingly, in human tuberculosis, lack of skin test reactivity in some individuals has been associated with an absence of lymphocyte skin-selective homing receptors.

The possible role of such a mechanism has not been investigated for bovine tuberculosis [3].

Infection in "closed" herds can occur and the source of infection is not always identified. Perumaalla et al.[10] reported that among those herds there still existed a high rate of infection that could only be explained by recrudescence and persistence within the herd. The authors concluded that the infections in herds might be attributed to the failure of current diagnostic testing to detect animals harboring the organism.

The isolation of *Mycobacterium bovis* from respiratory secretions of skin tested negative cattle has been reported, and those animals exhibited responses to mycobacterial antigens in *in vitro* assays[11]. Since those animals cannot be diagnosed by intradermal tests but may represent important threat to the success of the eradication program, their correct identification becomes essential.

The use of ELISA to identify anergic cattle has been reported by Placket et al. [5]. The authors also discussed that since such cattle may have a heavy bacterial load and are very likely to infect others their removal is essential to the success of an eradication campaign. Nevertheless, despite of this alert, ELISA testing did not become a routine in tuberculosis control programs and few references can be found referring to its use to identify anergic cows. Our group in a previous report[6] in which an in-house ELISA was evaluated and recommended as a herd investigation method developed similar considerations. Its use to identify anergic cattle has been suggested but no field cases utilizing the method in specific field situations have been described.

In this study we could observe the impact of the presence of skin test-negative cattle on the herd. In two herds, an important delay on the eradication of the disease has been observed, and particularly for Herd B, severe economic losses have been observed. After recovering *M.bovis* from tissues and lesions, we concluded that in both cases, the anergic cows had advanced tuberculosis. They were the most probable source of

continuing infection and responsible for the maintenance of the disease in their herds. Therefore, the application of ELISA was extremely advantageous to identify infected cows enabling their separation from the herd and assisting with disease eradication. Without the use of ELISA tests those cows would probably stay in their herds perpetuating the disease and the eradication of the disease in those herds would become impossible.

In conclusion, we alert veterinarians about important damages to the tuberculosis control program when an anergic animal is present on the herd. In cases where unexpected cases are identified after the elimination of the skin test-reactive cattle, we suggest the use of ELISA as a valuable complementary tool in order to identify possible anergic cows that may be acting as reservoirs of the agent on the herd.

Acknowledgements

O uso de Elisa como ferramenta complementar para o controle da tuberculose bovina no Brasil

Resumo

Palavras-chave:
Tuberculose.
Bovinos.
Anérgicos.
ELISA.

A detecção de animais infectados é um dos mais importantes fatores envolvidos no controle da tuberculose e, com algumas variações, é realizado através de testes intradérmicos de tuberculinização. No entanto, animais negativos aos testes intradérmicos podem estar infectados e representam uma importante ameaça aos programas de erradicação da tuberculose. Apesar deste conhecido fenômeno, o uso de ELISA não é rotina nestes programas. Nosso objetivo foi o de descrever experiências do uso a campo de ELISA na detecção de animais anérgicos no Rio de Janeiro, Brasil. Dentre 18 rebanhos envolvidos em um programa de controle da tuberculose, dois apresentaram animais infectados negativos aos testes intradérmicos, o que atrasou e comprometeu o sucesso do programa, com severas perdas econômicas. A infecção nestes animais foi identificada através de ELISA e confirmada pelo isolamento de *M.bovis* nas lesões pulmonares. Desta forma foram considerados como a mais provável fonte de infecção e responsáveis pela manutenção da enfermidade nos rebanhos. Sem o uso de testes sorológicos como ELISA estes animais provavelmente permaneceriam nos rebanhos perpetuando a infecção e a erradicação da enfermidade seria impossível. Em conclusão, sugere-se o uso de ELISA como uma valiosa ferramenta complementar para identificar animais anérgicos que possam atuar como reservatórios do agente nos rebanhos.

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